communications Journal of proteome research

Application of a Novel Protein Biochip Technology for Detection and Identification of Rheumatoid Arthritis Biomarkers in Synovial Fluid

Takafumi Uchida,*,† Ai Fukawa,‡ Mai Uchida,§ Kyosuke Fujita,|| and Kenji Saito‡

Institute of Development, Aging and Cancer, Tohoku University, Sendai, Japan, Ciphergen Biosystems Co., Kamakura., Japan, Hokkaido University, Sapporo, Japan, and Saitama City Hospital, Saitama, Japan.

Received May 15, 2002

Abstract: We compared protein profiles of the synovial fluid of patients with rheumatoid arthritis and osteoarthritis by using surface-enhanced laser desorption/ionization mass spectrometry technology. With this approach, we identified a protein expressed specifically in the synovial fluid of the patients with rheumatoid arthritis. During the investigation, we found several reproducible and discriminatory biomarker candidates for distinction between rheumatoid arthritis and osteoarthritis. Among these candidates, a 10 850 Da protein peak was the clearest example of a single signal found specifically in the rheumatoid arthritis samples. This candidate was purified using a size-exclusion spin column followed by gel electrophoresis and subsequently identified by peptide mapping and post-source decay (PSD) analysis. The results clearly indicate that the protein is myeloid-related protein 8, which was verified by the enzyme immunoassay. It is known that the myeloid-related protein 8 level in serum and synovial fluid is related to disease activity in juvenile rheumatoid arthritis. The results suggest that the ProteinChip platform is useful to detect and identify protein biomarkers expressed specifically in diseases or in some stage of diseases.

Keywords: ProteinChip system • rheumatoid arthritis • myeloid related protein 8 • biomarkers

Introduction

The study of mRNA expression was rapidly accelerated with the use of DNA microarray technology. However, it has been shown that the level of RNA expression does not always correlated with protein expression in eukaryotes.[1,2] Furthermore, the level of protein expression is more directly related to the physiological state of cells. In this respect, discovery research on factors involved in biological function, disease markers, and drug targets requires the study of protein expression.

Although 2D gel electrophoresis is currently the most widely used tool for the analysis of protein expression, it has disadvantages with regard to throughput, reproducibility, and sensitivity.[3] Recently, "protein biochip" technology is beginning to mature,[4] and it is expected to be one of the most popular technologies for rapid and sensitive protein expression analysis in the proteomics era. In this study, we evaluated the ProteinChip platform version of protein biochip technology as a tool for detecting and identifying proteins differentially expressed in disease.

We applied the ProteinChip technology to search for and identify proteins that were differentially expressed in the synovial fluid of the patients with rheumatoid arthritis (RA) and osteoarthritis (OA). OA is caused by the degeneration of cartilage, so OA is often called degenerative joint disease. RA is caused by inflammation of the synovium. Overgrowth of synovial cells induces an increase in secreted proteases, receptor activator of NF-κB, IL-6, and invasion of lymphocytes and macrophages, which causes inflammation of the synovium and degradation of cartilage.[5] Clear diagnosis of RA in early stages is very important, in view of determining the timing and amount of chemotherapy by immunosuppressive drugs. So far, the most popular diagnosis of RA is the measurement of RA factor, or the antibody against the Fc region of IgG.[6] The inflammation markers, or C-reactive protein and erythrocyte sedimentation rates, are also used to monitor the disease state of RA.[7] However, the false positive rate is relatively high with these measurements. In this sense, early diagnosis of RA still remains a challenge in the clinical setting. If a specific marker for RA can be found, then it is likely to prove very useful for the diagnosis and treatment of patients with RA.

In this study, we searched for a marker for RA by surface-enhanced laser desorption/ionization (SELDI) mass spectrometry analysis of synovial fluid. We identified a protein and showed that SELDI mass spectrometry analysis is a useful method for identification of disease markers.

Material and Methods

Protein Profiling. We used the synovial fluids that were removed for treatment. Synovial fluids were removed by aspiration with a small-gauge needle from the 12 patients with rheumatoid arthritis and 4 patients with osteoarthritis. (K.F. in Saitama City Hospital obtained the samples with the permission of the patients.) The synovial fluids containing proteins (20−45 μg) were diluted 1/10 with PBS.

The samples were analyzed with two kinds of chips; H4 (C16 hydrophobic surface) and SAX2 (strong anion exchange surface)

* To whom correspondence should be addressed. Takafumi Uchida, Ph.D., Department of Pathology, Institute of Development, Aging and Cancer, Tohoku University, 4-1 Seiryo, Aoba, Sendai, Miyagi 980-8575, Japan. Phone: 81-22-717-8511. Fax: 81-22-717-8512. E-mail: uchidat@idac.tohoku.ac.jp.

† Institute of Development, Aging and Cancer, Tohoku University.

‡ Ciphergen Biosystems Co.

§ Hokkaido University.

|| Saitama City Hospital.

10.1021/pr025531w CCC: $22.00

Published on Web 09/17/2002

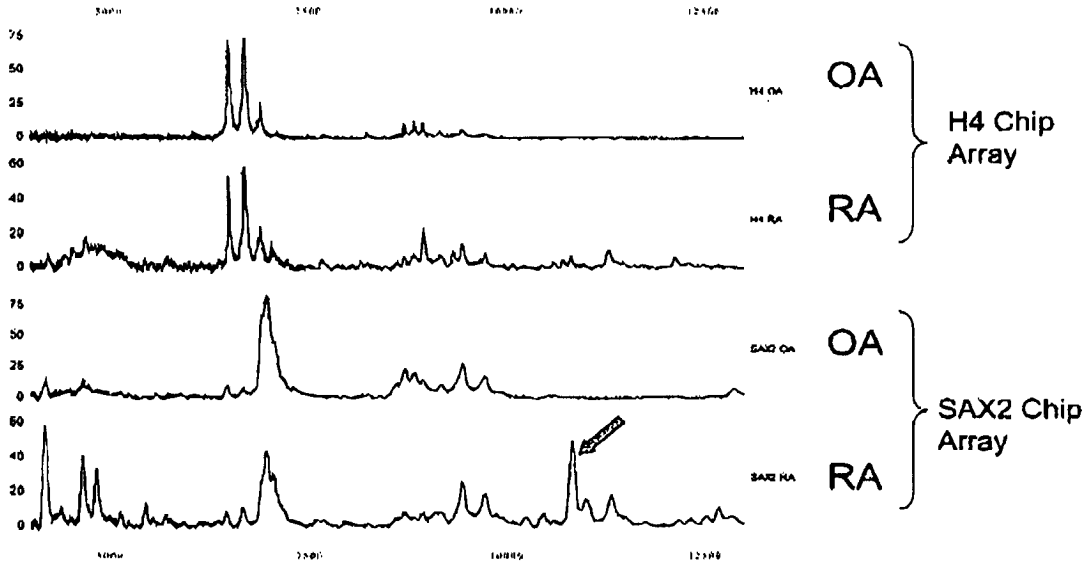


Figure 1. Protein profiling of synovial fluid from OA and RA patients. Synovial fluid samples from RA and OA patients were analyzed using hydrophobic (H4) and strong anion exchange (SAX2) ProteinChip Array. Several proteins were specifically observed only in the RA samples. Among them, a 10 850 Da protein from the SAX2 array profile appeared very specific in synovial fluid from RA patients.

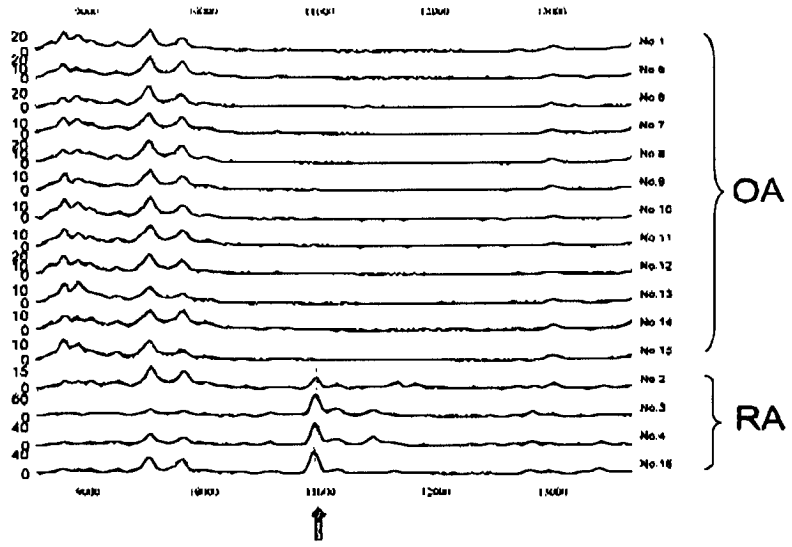


Figure 2. Comparison of RA and OA synovial fluid with SAX2 chip array. Evaluation of the 10 850 Da protein as a marker candidate. Appearance of the 10 850 Da protein in only the synovial fluid of RA patients was further confirmed with samples from 8 OA and 4 RA patients. The 10 850 Da protein appeared in all synovial fluid samples from RA patients. On the other hand, this marker was not detected in the synovial fluid from OA patients.

ProteinChip arrays (Ciphergen Biosystems, Inc., Fremont, CA). H4 (C16 hydrophobic surface): 0.5 μL of acetonitrile was applied to each spot of an H4 ProteinChip Array, and 1 μL of diluted sample was added before the acetonitrile evaporated to dryness. The array surface was then air-dried at room temperature. Next, the array surface was washed twice with 5 μL of wash buffer (1M Urea, 0.125% CHAPS, 0.25M NaCl, and 50mM HEPES) by pipetting. The array was finally rinsed quickly with distilled water. SAX2 (strong anion exchange surface): a 5 μL portion of diluted sample was applied to each spot of a SAX2 ProteinChip array. The sample was incubated for 15 min to allow interaction with the surface. The array surface was washed twice with 5 μL of wash buffer (20mM Tris-HCl, and 5mM NaCl (pH9)) and rinsed with 5 μL of distilled water.

After the array surfaces were air-dried, 0.5 μL of saturated sinapinic acid in 50% acetonitrile and 0.5% TFA was applied

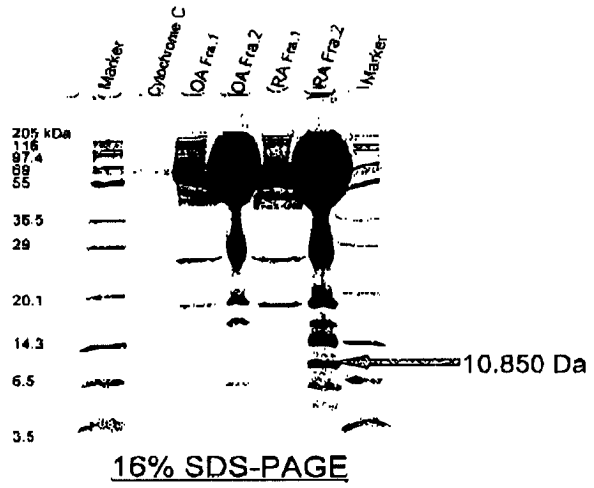


Figure 3. Separation of the 10 850 Da protein by SDS−PAGE. The 10 850 Da protein was finally separated by SDS−PAGE. For both OA and RA samples, Fractions 1 and 2 of the 70 K size-exclusion spin column were separated by SDS−PAGE. Proteins were stained by Coomasie Brilliant Blue. The 10 850 Da protein was clearly observed in fraction 2 of the RA sample. The corresponding band was not observed in OA sample. Lane 1 and 7: molecular markers. Lane 3: Fraction 1 of OA sample. Lane 4: Fraction 2 of OA sample. Lane 5: Fraction 1 of RA sample. Lane 6: Fraction 2 of RA sample.

twice to the surface. Mass analysis was performed with a ProteinChip Reader (Model PBS II, Ciphergen Biosystems) at room temperature. The energy settings on the instrument calibrations are as follows: H4 chip: detector voltage, 1800; sensitivity, 10; laser intensity, 245. SAX2 chip: detector voltage, 1800; sensitivity, 10; laser intensity, 260.

Purification. Synovial fluid from OA and RA patients was fractionated using a 70 K size-exclusion spin column (Ciphergen Biosystems, Palo Alto). The samples were diluted 1/5 with 20 mM Tris-HCl, pH8. A 40 μL portion of the diluted sample was added to the column and centrifuged (2000 rpm, 2 min: Fraction 1). Then, a 40 μL portion of the elution buffer (20 mM Tris-HCl, pH8) was added to the column and centrifuged again (Fraction 2). The last process was repeated twice (Fraction 3 and Fraction 4). Each fraction was analyzed with the ProteinChip Reader to check the presence of the target protein.

The fraction containing the target protein was concentrated by speed-vac and subjected to separation by 16% SDS−PAGE. The proteins were stained by Coomasie Brilliant Blue. The band at 10 850 Da that corresponded to the molecular weight of the target protein was excised from the gel.

Identification. We identified the biomarker candidate by two distinct methods. First, the target protein was identified by peptide mapping.[8] The target protein was digested in-gel with trypsin overnight at 37 °C. A part of the gel displaying no band (control digest) was also excised and digested with trypsin as a control. The molecular weight of each peptide was measured with a ProteinChip reader using H4 ProteinChip array. The ProteinChip reader was calibrated with β-endorphin and two peptides derived from digestion of typsin. The mass error was 1.0 Da. The molecular weights of peptides derived only from the target protein were collected and analyzed with ProFound software (http://prowl.rockefeller.edu/cgi-bin/ProFound) for protein identification through peptide mass fingerprinting. Sequence information of one of the tryptic peptides derived from the target protein was further analyzed by Post Source Decay (PSD) analysis.[9] The fragmentation pattern of the peptide was gathered by MALDI-TOF MS with PSD function.

The sequence was determined by analyzing the pattern with Mascot software (Matrix Science, London, UK).

Measurement of MRP8. MRP8 in the synovial fluid was measured by enzyme immunoassay following the vendor's manual (MA Biomedicals AG, Switzerland). The test was performed using a one-step noncompetitive sandwich assay. The samples and standard MRP8 solution were added into the 96 wells coated with anti-MRP8 antibody and the plate was incubated at 37 °C for 30 min. After the plate was washed with water several times, peroxidase-labeled anti-MRP8 antibody was added and incubated for 30 min at 37 °C. After the plates were washed several times, tetramethylbenzidin solution was added to each well, and the plates were incubated for 15 min, at room temperature. The reaction was stopped with 1 M sulfuric acid, and the absorbance was measured at 450 nm with a reference wavelength of 630 nm.

Results and Discussion

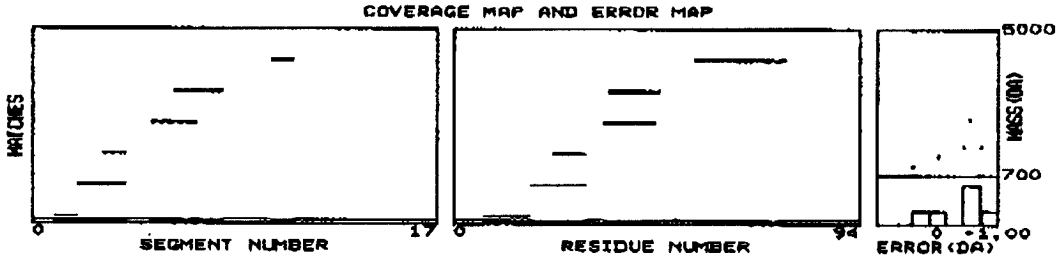
We identified biomarkers specific for RA by comparing the synovial fluid of RA and OA patients. Several RA specific peaks appeared differentially regulated on both the SAX2 (3412, 3492, 4684, 4845, 10 850, 11 417, 12 786, 15 981, 38 999) and H4 arrays (H4: F3360, 3432, 3476, 3700, 4122, 4616, 10 833, 11 320, 14 000, 26 708) (Figure 1). Among them, the peak at 10 850 Da on the SAX2 array looked most specific and strong (Figure 1). A variety of samples including RA and OA were examined with SAX2 array reproducible (Figure 2). Therefore, we purified the protein from the synovial fluid of RA patients. As a control, an OA sample was treated following the same procedure. The proteins were fractionated using a 70 K size-exclusion spin column, and each fraction was checked with the ProteinChip reader. The fraction that contained the target protein was concentrated. Proteins in the fraction were separated with 16% SDS−PAGE. The proteins were stained with Coomasie Brilliant Blue. The band corresponding to 10 850 Da (Figure 3) was excised and digested by trypsin treatment. The specific peaks of the digested sample were analyzed with Profound software. The results of the database search suggested that the protein could be MRP8 that is the same protein as S100 calcium- binding protein and cystic fibrosis antigen (Figure 4). PSD analysis was performed for the confirmation of this identification. The 10 850 Da protein was also digested by trypsin and analyzed with MALDI-TOF MS containing PSD function and the degradation pattern was analyzed by Mascot software. The protein sequence determined by this PSD analysis showed that the protein is migration inhibitory factor−related protein 8 (MRP8), which is the same protein as S100 calcium-binding protein A8 or cystic fibrosis antigen (Figure 5). The results matched the results obtained by peptide mass fingerprinting. Taken together, we concluded that the protein is MRP8. To further confirm the results, we measured the amount of MRP8 in the synovial fluid quantitatively by enzyme immunoassay. The amount of MRP8 determined by enzyme immunoassay related to the height of the 10 850 Da protein peak in the SELDI mass spectrometry analysis is MRP8 (Figure 6), which suggests that 10 850 Da protein is MRP8.

MRP8 belongs to the S100 family of calcium binding proteins.[10] MRP8 exists as a heterodimer with MRP14 in several

(a)

Rank	Probability	Protein	GI number	molecular weight (kDa)
1	4.2E-01	cystic fibrosis antigen [Homo sapiens]	gi225541	11
2	4.2E-01	S100 calcium-binding protein A8 [Homo sapiens]	gi4506771	11
3	1.7E-01	human calgranulin A (migration inhibitory factor-related protein 8) (MRP-8)	gi115442	11
4	2.2E-07	CENP-E protein [Homo sapiens]	gi382658	312
5	2.2E-07	centromere protein E [Homo sapiens]	gi4502781	312

gi225541 cystic fibrosis antigen [Homo sapiens]
Digestion chemistry: Trypsin
Number of measured peptides: 8
Number of matched peptides: 5
Coverage of protein sequence: 62%



(b)

Measured Mass (M/Average)	Computed Mass	Error (Da)	Residues (From)	Residues (To)	Missed Cut	Cys	Met
962.69	963.07	-0.38	24	31	0	0	0
1272.50	1272.47	0.03	8	18	0	0	0
1564.30	1563.84	0.46	36	47	1	1	0
1564.30	1563.84	0.46	37	48	1	1	0
1568.58	1567.81	0.77	19	31	1	0	0
2392.33	2391.75	0.58	57	77	0	0	0

Figure 4. Peptide mapping of the 10 850 Da protein. Molecular weights of tryptic peptides from the 10 850 Da protein were collected with SELDI-TOF mass spectrometry. Peptide mapping analysis was performed with ProFound software (a). Three proteins [migration inhibitory factor-related protein 8 (MRP8), S100 calcium-binding protein A8 and cyctic fibrosis antigen] predicted with high confidence scores were the same protein. The coverage map and error map of the most matched candidate MRP8 was summarized in (b), again using ProFound software.

1. gi|115442 calgranulin A (migration inhibitory factor-related protein 8) (MRP-8) [Homo sapiens]
Mass: 10828, Score: 72
Observed mass: 1273.00, Mr(expt): 1271.99, Mr(calc): 1271.69, Delta mass: 0.30
Start-End: 8-18, Miss: 0, Ions 49
Peptide: ALNSIIDVYHK

2. gi|4506771 S100-calcium binding protein A8 [Homo sapiens]
Mass: 10931, Score: 72
Observed mass: 1273.00, Mr(expt): 1271.99, Mr(calc): 1271.69, Delta mass: 0.30
Start-End: 8-18, Miss: 0, Ions 49
Peptide: ALNSIIDVYHK

3. gi|225541 cystic fibrosis antigen [Homo sapiens]
Mass: 10946, Score: 72
Observed mass: 1273.00, Mr(expt): 1271.99, Mr(calc): 1271.69, Delta mass: 0.30
Start-End: 8-18, Miss: 0, Ions 49
Peptide: ALNSIIDVYHK

Figure 5. PSD analysis of 10 850 Da protein. One tryptic peptide from the 10 850 Da protein was selected and subjected to MALDI analysis with PSD function. By analyzing the degradation pattern with Mascot software, the same protein, MRP8, predicted by peptide mapping was identified again.

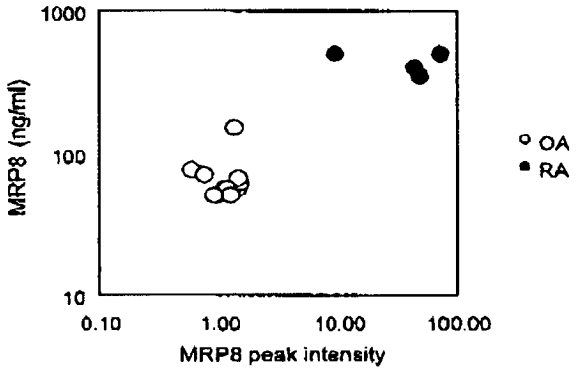


Figure 6. Comparison of ProteinChip array and EIA results. Measurement of MRP8 by enzyme immunoassay (EIA). The amount of MRP8 measured by enzyme immunoassay is plotted against peak intensity obtained by the ProteinChip Biology System. There was good correlation between the results obtained by enzyme immunoassay and ProteinChip analysis.

inflammatory diseases.[11-14] The MRP8/MRP14 heterodimer is thought to be involved in the process of inflammation, including the transmigration process of leukocytes and transport of

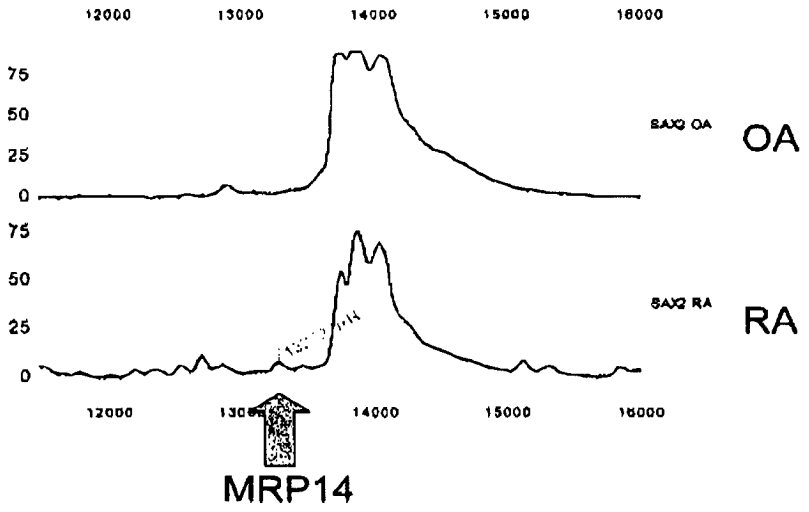


Figure 7. Peak corresponding to MRP14. The peak corresponding to the molecular weight of MRP14 was observed only in synovial fluid from RA patients.

arachidonic acid to target cells.[15-18] In our study, a peak whose molecular weight corresponds to MRP14 was also observed only in synovial fluid of RA patients (Figure 7). This observation is consistent with the previous reports that showed the existence of MRP8/MRP14 in serum and/or synovial fluid of RA patients.[19] Our results suggested that the amount of MRP8 itself could be a sufficient marker for distinguishing rheumatoid arthritis from other joint diseases such as osteoarthritis. The patients with rheumatoid arthritis tended to show high rates of CRP. Combining MRP8 with other conventional markers, such as CRP and RA factor will lead to more precise diagnosis of rheumatoid arthritis. Moreover, there were several other peaks that were observed only in the synovial fluid of RA patients (Figure 1). Pattern analysis in combination with these proteins would enable more reliable and detailed monitoring of the RA disease state. To confirm this possibility, a study with more samples from patients with several stages of RA and other inflammatory diseases would be necessary.

Biomarker discovery is often a tedious and time-consuming process. We found MRP8 and other marker candidates for RA within several hours and could rapidly validate the results by increasing the number of samples. In conclusion, we confirmed that the ProteinChip platform is a promising technology for biomarker discovery, and it offers the possibility of accelerating the process of discovering disease markers.

Acknowledgment. We thank Morito Takahashi for technical help, and Akira Ohashi, Fukumoto M., and Takeuchi T. for helpful discussions throughout this research. This work was supported by Grant No. 13460148-00 from the Ministry of Education, Science, Sports, and Culture of Japan to T.U.

References

(1) Gygi, P. S.; Rochon, Y.; Franza, B. R.; Aebersold, R. *Mol. Cell. Biol.* **1999**, *19*, 1720−1730.
(2) Ideker, T.; Thorsson, V.; Ranish, A. J.; Christmas, R.; Buchler, J.; Eng, K. J.; Bumgarner, R.; Goodlett, R. D.; Aebersold, R.; Hood, L. *Science* **2001**, *292*, 929−934.
(3) Jenkins, R. E.; Pennington, S. R. *Proteomics* **2001**, *1*, 13−29.
(4) Merchant, M.; Weinberger, R. S. *Electrophoresis* **2000**, *21*, 1164−1167.
(5) Cunnane, G.; Hummel, K. M.; Muller-Ladner, U.; Gay, R. E.; Gay, S. *Arch. Immunol. Ther. Exp.* **1998**, *46*, 1−7.
(6) Scott D. L. *Rheumatology* **2000**, *39*, 24−29.
(7) van Zeben D.; Breedveld F. C. *J. Rheumatol.* **1996**, *44*, 31−33.
(8) Mortz, E.; Vorm, O.; Mann, M.; Roepstorff, P. *Biol. Mass Spectrom.* **1994**, *23*, 249−261.
(9) Spengler, B.; Kirsch, D.; Kaufmann, R. *J. Phys. Chem.* **1992**, *96*, 9678−9684.
(10) Odink, K.; Cerletti, N.; Brüggen, J.; Clerc, G. R.; Tarcsay, L.; Zwadlo, G.; Gerhards, G.; Schlegel, R.; Sorg, C. *Nature* **1987**, *330*, 80−82.
(11) Bhardwaj, R. S.; Zotz, C.; Zwadlo-Klarwasser, G.; Roth, J.; Goebeler, M.; Mahnke, K.; Falk, M.; Meinardus-Hager, G.; Sorg, C. *Eur. J. Immunol.* **1992**, *22*, 1891−1897.
(12) Burkhardt, K.; Radespiel-Troger, M.; Rupprecht, H. D.; Goppelt-Strube, M.; Riess, R.; Renders, L.; Hauser, I. A.; Kunzendorf, U. *J. Am. Soc. Nephrol.* **2001**, *12*, 1947−1957.
(13) Roth, J.; Teigelkamp, S.; Welke, M.; Grun, L.; Tummler, B.; Sorg, C. *Immunobiology* **1992**, *186*, 304−314.
(14) Schmid, K. W.; Lugering, N.; Stoll, R.; Brinkbaumer, P.; Windo, G.; Domschke, W.; Bocker, W.; Sorg, C. *Hum. Pathol.* **1995**, *26*, 334−337.
(15) Eue, I.; Pietz, B.; Storck, J.; Klempt, M.; Sorg, C. *Int. Immunol.* **2000**, *12*, 1593−1604.
(16) Klempt, M.; Melkonyan, H.; Nacken, W.; Wiesmann, D.; Holtkemper, U.; Sorg, C. *FEBS Lett.* **1997**, *408*, 81−84.
(17) Kerkhoff, C.; Eue, I.; Sorg, C. *Pathobiology* **1999**, *67*, 230−232.
(18) Hessian, P. A.; Edgeworth, J.; Hogg, N. *J. Leukoc. Biol.* **1993**, *53*, 197−204.
(19) Frosch, M.; Strey, A.; Vogl, T.; Wulffraat, N. M.; Kuis, W.; Sunderkotter, C.; Harms, E.; Sorg, C.; Roth, J. *Arthritis Rheum.* **2000**, *43*, 628−637.

PR025531W